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The presence of KRAS, PPP2R1A and ARID1A mutations in 101 Chinese samples with ovarian endometriosis

Yang Zou[a,b,1], Jiang-Yan Zhou[a,c,1], Jiu-Bai Guo[a,c], Li-Qun Wang[a,c], Yong Luo[a,b], Zi-Yu Zhang[a,b], Fa-Ying Liu[a,b], Jun Tan[d], Feng Wang[a,b], Ou-Ping Huang[a,c,*]

[a] Key Laboratory of Women's Reproductive Health of Jiangxi Province, Jiangxi Provincial Maternal and Child Health Hospital, Nanchang, Jiangxi 330006, China
[b] Central Laboratory, Jiangxi Provincial Maternal and Child Health Hospital, Nanchang, Jiangxi 330006, China
[c] Department of Gynecology, Jiangxi Provincial Maternal and Child Health Hospital, Nanchang, Jiangxi 330006, China
[d] Reproductive Medicine Center, Jiangxi Provincial Maternal and Child Health Hospital, Nanchang, Jiangxi 330006, China

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ABSTRACT

Endometriosis is a potential premalignant disorder. The underlying molecular aberrations, however, are not fully understood. A recent exome sequencing study found that 25% (10/39) of deep infiltrating endometriosis harbored cancer driver gene mutations. However, it is unclear whether these mutations also exist in ovarian endometriosis. Here, a total of 101 ovarian endometriosis samples were analyzed for the presence of these gene mutations, including KRAS, PPP2R1A, PIK3CA and ARID1A. In addition, 6 other cancer-associated genes (BRAF, NRAS, HRAS, ERK1, ERK2 and PTEN) were also analyzed. In total, four somatic mutations were identified in three out of 101 ovarian endometriotic lesions (4%, 4/101), including a KRAS p.G12V, a PPP2R1A p.S256F and two ARID1A nonsense mutations (p.Q403* and p.G1926*); while no mutations were identified in the remaining 7 genes (BRAF, NRAS, HRAS, ERK1, ERK2, PTEN and PIK3CA). Note that the KRAS G12V and ARID1A Q403* mutations co-occurred in a 36-year-old sample who had a high serum CA125 (308.4 U/mL) and a late menarche age (18-year-old). Additionally, no mutations in any of the 10 genes were identified in either the healthy eutopic endometrial tissues from 85 control individuals without endometriosis, or in 62 healthy ovarian tissues from ovarian cysts samples (without endometriosis). Our study revealed, for the first time, the presence of classical cancer driver gene mutations in ovarian endometriosis. Furthermore, the co-occurrence of KRAS and ARID1A mutations was identified in a single individual for the first time. The observations of cancer driver gene mutations in our ovarian endometriosis samples, together with several prior observations, further support the notion that endometriosis is a premalignant disorder.

1. Introduction

Endometriosis is a common and enigmatic gynecologic condition characterized by the presence of the endometrial gland and stroma outside the uterus. This condition affects approximately 6–10% of women in child-bearing age [1]. It can be subdivided into three distinct pathological entities, peritoneal superficial endometriosis, ovarian endometriosis and deep infiltrating endometriosis, according to the etiological and morphological differences [2]. Prior studies have found the fact that specific subtypes of ovarian carcinoma are closely associated with endometriosis [3,4].

Epidemiological and molecular data also imply that endometriosis might be a premalignant lesion in specific subtypes of ovarian carcinoma [5,6]. Most recently, an exome sequencing study has found that samples with deep infiltrating endometriosis harbored frequent somatic mutations in multiple cancer driver genes, including KRAS (KRAS proto-oncogene, GTPase), PPP2R1A (protein phosphatase 2 scaffold subunit A alpha), PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha) and ARID1A (AT-rich interaction domain 1A) [7]. Similarly, an earlier large-scale sequencing effort had revealed that the endometriotic lesions from ovarian endometriosis harbored somatic mutations in multiple genes [8]. Considering the fact that the three forms of endometriosis shared some clinical and molecular aberrations [9,10] and the relatively small sample size analyzed in the prior study (n = 16) [8], it remains largely unclear whether these frequently observed cancer driver gene mutations in deeply infiltrating

endometriosis also exist in ovarian endometriosis.

To test this hypothesis, we analyzed a total of 101 Chinese samples with ovarian endometriosis for the presence of somatic mutations in the four cancer driver genes (KRAS, PPP2R1A, PIK3CA and ARID1A) [7]; in addition, 6 other cancer-associated genes, BRAF (B-Raf proto-oncogene, serine/threonine kinase), NRAS (NRAS proto-oncogene, GTPase), HRAS (HRas proto-oncogene, GTPase), ERK1 (mitogen-activated protein kinase 3), ERK2 (mitogen-activated protein kinase 1) and PTEN (phosphatase and tensin homolog), were also analyzed. In total, we identified four somatic mutations in KRAS, PPP2R1A and ARID1A genes in three out of 101 ovarian endometriotic lesions; among which, the KRAS G12 V and ARID1A Q403* mutations co-occurred in a single sample.

2. Materials and methods

2.1. Patients

The endometriotic lesions and paired peripheral blood leukocytes were collected from a cohort of 101 ovarian endometriosis samples undergoing hysterectomy in Jiangxi Provincial Maternal and Child Health Hospital (Nanchang, China). In addition, the healthy eutopic endometrial tissues from a total of 85 control samples without endometriosis, as well as healthy ovarian tissues from a group of 62 ovarian cysts samples (without endometriosis), were also recruited. All of these samples were cancer-free. The present study was performed according to the tenets of the Helsinki Declaration and approved by the Institutional Review Board of Jiangxi Provincial Maternal and Child Health Hospital. Each patient signed an informed consent prior to the inclusion in the study.

2.2. Clinical data

Patient's age at diagnosis, age at menarche was obtained at the time of sampling. In addition, the clinical data of each sample, including serum hemoglobin, free triiodothyronine (FT3), thyroid stimulating hormone (TSH), free thyroxine (FT4), cancer antigen 125 (CA125), carcinoembryonic antigen (CEA), α-fetoprotein (AFP) and squamous cell carcinoma antigen (SCCA), was quantified on day 3 of the menstrual cycle by radioimmunoassay, as described previously [11]. An automated hematology analyzer XN-3000 (Sysmex Corporation, Kobe, Japan) was used to count the numbers of white blood cell, eosinophil granulocyte, lymphocyte, neutrophil granulocyte, mononuclear cell, platelet and neutrophil granulocyte proportion (Table 1).

2.3. DNA extraction and mutation analysis

The genomic DNA was extracted from the endometriotic lesions and paired peripheral blood leukocytes with Omega Tissue or Blood DNA kit (OMEGA Bio-tek Inc., Doraville, GA, USA), respectively. The DNA was then used in PCR reactions to amplify the potential mutational hotspot regions of KRAS, PPP2R1A, PIK3CA, BRAF, NRAS, HRAS, ERK1, ERK2 and PTEN genes, as well as the entire coding region and corresponding intron/exon boundaries of the ARID1A gene with a set of primer pairs (Table 2). In brief, the PCR reactions were performed in a final volume of 50 μL, containing 2.5 mM dNTPs (Takara Biotechnology), 2.5 mM of MgCl$_2$ (Takara Biotechnology), 1 U LA Taq (Takara Biotechnology), 0.5 μM (10 μM) of each primer. The PCR protocol included an initial activation step at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 50–60 °C (Table 2) for 30 s and an extension step at 72 °C for 30 s. The PCR products were purified with a DNA purification kit (Tiangen, Beijing, China) and sequenced in both directions on ABI 3730 Automatic Capillary DNA Sequencer (Applied Biosystems, USA). For the mutations identified in endometriotic lesions, the somatic mutations were confirmed by sequencing the paired peripheral blood leukocytes.

Table 1
The clinical characteristics of 101 samples with ovarian endometriosis in the present study.

Clinical features	Median	Range
Age in years (years)	32	21–50
Median age of menarche(years)	14	10–18
Hemoglobin(g/L)	121.5	74.00 − 142.00
TSH(mIU/mL)	2.15	0.75 − 6.44
FT3(pg/mL)	3.05	1.25 − 3.85
FT4(ng/dL)	1.28	0.92 − 1.66
AFP(ng/mL)	2.01	0.93 − 15.13
CEA(ng/mL)	0.92	0.21 − 2.90
CA125(μ/mL)	63.52	11.58–342.20
SCC(ng/mL)	1.14	0.34 − 5.08
Whitebloodcellcount(x10^9)	6.04	3.11–16.58
Lymphocyte cell count(x10^9)	1.87	0.41 − 2.98
Eosinophil granulocyte(x10^9)	0.09	0.01 − 0.60
Mononuclear cell count(x10^9)	0.42	0.12 − 1.35
Neutrophil granulocyte cell count(x10^9)	3.16	1.49 − 15.00
Platelet(x10^9)	199.00	95.00 − 415.00
Neutrophil cell proportion(%)	57.90	35.70–94.70

TSH, thyroid stimulating hormone; FT3, free triiodothyronine; FT4, free thyroxine; AFP, α-fetoprotein; CEA, carcinoembryonic antigen; CA125, cancer antigen 125; SCCA, squamous cell carcinoma antigen.

2.4. Evolutionary conservation analysis of PPP2R1A p.S256F mutation

Evolutionary conservation analysis was used to infer the potential pathogenicity of the identified mutations with 19 species from GenBank, including Homo sapiens (GenBank accession NP_055040.2), Pan troglodytes (XP_016789826.1), Pongo abelii (NP_001126285.1), Macaca mulatta (NP_001244851.1), Nomascus leucogenys (XP_012365376.1), Callithrix jacchus (XP_002762479.1), Rattus norvegicus (NP_058735.1), Mus musculus (NP_058587.1), Sus scrofa (NP_999189.1), Cricetulus griseus (XP_007648156.1), Tribolium castaneum (NP_001335982.1), Canis lupus familiaris (XP_850993.1), Bos taurus (NP_001032554.1), Ovis aries (XP_011950091), Anolis carolinensis (XP_003225135.3), Felis catus (XP_003997500.1), Odobenus rosmarus divergens (XP_004415541.1), Xenopus laevis (NP_001081031.1) and Danio rerio (NP_001299846.1).

2.5. Statistical analysis

The statistical analysis was performed with the statistical software package SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA). P-value for comparisons of mutation frequencies of cancer driver genes between the present and the prior studies [7,8] was calculated with Fisher's exact test. All tests were two-sided and a P-value < 0.05 was considered statistically significant.

3. Results

3.1. Patient characteristics

The median age of the patients was 32 years (range, 21–50) and the median age at menarche was 14 years (range, 10–18). The detailed clinical data, including hemoglobin, TSH, FT3, FT4, AFP, CEA, CA125, SCCA and blood cell counts, was summarized in Table 1.

3.2. KRAS, PPP2R1A, ARID1A mutations in ovarian endometriosis

A total of four somatic mutations were identified in three out of 101 ovarian endometriotic lesions (4%, 4/101), including a KRAS p.G12 V (c.35G > T) missense mutation, a PPP2R1A p.S256F (c.767C > T) missense mutation and two ARID1A nonsense mutations: p.Q403* (c.1207C > T) and p.G1926* (c.5776G > T) (Fig. 1); while no mutations were detected in the remaining 7 genes in our samples. Of note,

Table 2
The primer sequences for the mutation analysis of the cancer driver genes.

Gene	Potential hotspot mutations	Exon	Annealing temperature	Amplicon (bp)	Forward primer (5′-3′)	Reverse primer (5′-3′)
KRAS [23]	p.G12-G13	2	52 °C	162	ctgctgaaaatgactgaata	atggtcctgcaccagtaata
KRAS [23]	p.Q61	3	52 °C	132	ccagactgtgtttctcccttc	aaagaaagccctcccccagt
NRAS [23]	p.G12-G13	2	52 °C	118	gactgagtacaaactggtggtgg	gggcctcacctctcatggtg
NRAS [23]	p.Q61	3	52 °C	201	caagtggttatagatggtgaaacc	aagatcatcctttcagagaaaataat
HRAS [23]	p.G12-G13	2	52 °C	139	caggagaccctgtaggagga	tcgtccacaaaatggttctg
HRAS [23]	p.Q61	3	52 °C	194	tcctgcaggattcctaccgg	ggttcacctgtactggtgga
BRAF [23]	p.V600	15	52 °C	228	tgcttgctctgataggaaaatg	agcatctcagggccaaaaat
ERK1 [23]	p.E339	7	60 °C	246	ctgctctcactactgcaaaacc	tggcagcaggtatatctcagg
ERK2 [23]	p.E322	7	55 °C	231	ctgactcctgcccttccata	gggtggtagagacagcaagg
PPP2R1A	p.P179-R183; pS256-W257	5, 6	56 °C	575	tctgtgcttgctcctctct	gttccatcggcctaatgg
PIK3CA	p.E542-E545	9	58 °C	291	ccagagggaaaaatatgaca	gctttatttattccaata
PIK3CA	p.M1043-G1049	20	56 °C	426	catttgctccaaactgacca	tctaatgctgttcatggat
PTEN	p.R130G, p.R130Q	5	50 °C	557	ccgtatagcgtaaattcccaga	tctcagatccaggaagaggaa
ARID1A	–	1	58 °C	1218	ccgcgaggcccgcccgggcg	aggctgtggcccgctcac
ARID1A	–	2	60 °C	373	tattcagtggccagaggc	ctgtcaagaggcttggaag
ARID1A	–	3	54 °C	697	tactcatcatcagtgcat	tcaatcaatccagttatc
ARID1A	–	4	56 °C	262	agcctgcctggtttatca	attagctaaacttccaac
ARID1A	–	5, 6	56 °C	772	agaatctttctgcctaata	tgtcctaagcttcatggtc
ARID1A	–	7, 8	52 °C	1250	gagagcatttgttcgcat	agtgggtctataatacat
ARID1A	–	9, 10	55 °C	508	agctcagagtctaacct	tctagtccggtgtcata
ARID1A	–	11	58 °C	372	cactgctccagtcaagag	acatgaagccagtgag
ARID1A	–	12	52 °C	482	ctaagaactgtggttctac	actctctagtcagccaac
ARID1A	–	13, 14	57 °C	660	tgagagttaaacactgtc	tgcattatctcagaggat
ARID1A	–	15–17	56 °C	707	gtagattaccaggcttgtc	agtgagcttcttagacttca
ARID1A	–	18	55 °C	1034	tatgtccctgagtgcaga	tcaacctcatccagaatag
ARID1A	–	19	55 °C	358	tgtgttatcttcagagtag	agtactggcatggaagata
ARID1A	–	20 − 1	53 °C	978	ataattctgttcttaggcca	agcttgcccaggatgagca
ARID1A	–	20 − 2	60 °C	850	ataccattcgaagcctgtc	gagttcatcaacggtgata

the KRAS G12V and ARID1A Q403* mutations co-occurred in a 36-year-old sample who had a high serum CA125 (308.4 U/mL) and a late menarche age (18-year-old). The PPP2R1A-mutated sample was 25 years old with no history of pregnancy, who had an elevated serum CA 125 level (49.52 U/mL) and underwent menarche at age of 12; while the sample with ARID1A G1926* mutation was 27 years old and also diagnosed with uterine leiomyoma, whose serum CA125 was 97.45 U/mL and the menarche age was 15, no other obvious gynecological condition was observed. In addition, no mutations in any of the 10 genes were detected in either the healthy eutopic endometrial tissues from 85 control individuals without endometriosis, or in 62 healthy ovarian tissues from ovarian cysts samples (without endometriosis).

3.3. Evolutionary conservation analysis of PPP2R1A mutation

The evolutionary conservation analysis result suggested that the PPP2R1A S256F mutation changed a highly conserved serine at amino acid 256 to phenylalanine among the 19 vertebrate species from *Homo sapiens* to *Danio rerio* (Fig. 2).

4. Discussion

In the present study, a total of four somatic mutations in well-known cancer-associated genes were identified in three out of 101 ovarian endometriotic lesions, including a KRAS p.G12 V mutation, a PPP2R1A

p.S256F mutation and two ARID1A nonsense mutations p.Q403* and p.G1926*; while no mutations were detected in 7 other cancer-associated genes (PIK3CA, BRAF, NRAS, HRAS, ERK1, ERK2 and PTEN). Among these mutations, the KRAS G12V mutation was a well-known hotspot mutation in multiple human cancer types [12], the ARID1A Q403* mutation was also detected previously in 1 out of 15 esophageal adenocarcinomas [13] and 2 out of 69 urinary tract carcinomas [14,15]; while the ARID1A G1926* mutation was identified in 1 out of 147 rhabdomyosarcomas in a prior comprehensive genomic study [16]; for the PPP2R1A S256F mutation, it was also found in 1 out of 116 endometrial cancers [17] and the mutated amino acid located in the mutation hotspot regions of this gene [18]; the evolutionary conservation analysis result revealed that this mutation changed a highly conserved serine to phenylalanine in vertebrate species and thus might be a damaging mutation. Noted that no mutations in any of the 10 genes were identified in either the healthy eutopic endometrial tissues from 85 control individuals without endometriosis, or in 62 healthy ovarian tissues from ovarian cysts samples (without endometriosis). Taken together, these results suggested that these mutations identified in the current study might be positively involved in the pathogenesis of ovarian endometriosis. To the best of our knowledge, this is the first report revealing that ovarian endometriosis harbors somatic mutations in multiple cancer driver genes.

Similar to the prior observations, we also detected cancer driver gene mutations in our ovarian endometriosis samples, despite the
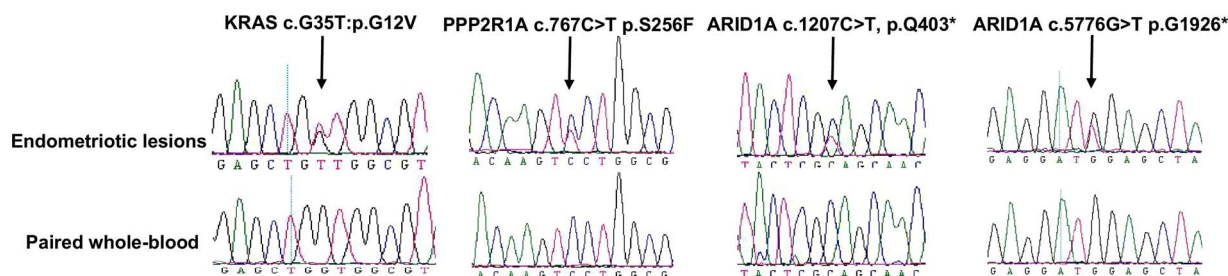

Fig. 1. The sequencing electropherograms of KRAS, PPP2R1A and ARID1A mutations, the arrow refers to locations of the mutations.

p.S256
↓

Homo sapiens (NP_055040.2)	R Q A A E D K S W R V R Y M V
Mus musculus (NP_058587.1)	R Q A A E D K S W R V R Y M V
Rattus norvegicus (NP_058735.1)	R Q A A E D K S W R V R Y M V
Bos taurus (NP_001032554.1)	R Q A A E D K S W R V R Y M V
Pan troglodytes (XP_016789826.1)	R Q A A E D K S W R V R Y M V
Macaca mulatta (NP_001244851.1)	R Q A A E D K S W R V R Y M V
Pongo abelii (NP_001126285.1)	R Q A A E D K S W R V R Y M V
Canis lupus familiaris (XP_850993.1)	R Q A A E D K S W R V R Y M V
Cricetulus griseus (XP_007648156.1)	R Q A A E D K S W R V R Y M V
Tribolium castaneum (NP_001335982.1)	R N C T G D T S W R V R Y M V
Sus scrofa (NP_999189.1)	R Q A A E D K S W R V R Y M V
Nomascus leucogenys (XP_012365376.1)	R Q A A E D K S W R V R Y M V
Callithrix jacchus (XP_002762479.1)	R Q A A E D K S W R V R Y M V
Ovis aries (XP_011950091)	R Q A A E D K S W R V R Y M V
Anolis carolinensis (XP_003225135.3)	R Q A A E D K S W R V R Y M V
Felis catus (XP_003997500.1)	R Q A A E D K S W R V R Y M V
Odobenus rosmarus divergens (XP_004415541.1)	R Q A A E D K S W R V R Y M V
Xenopus laevis (NP_001081031.1)	R Q A A E D K S W R V R Y M V
Danio rerio (NP_001299846.1)	R Q A A E D K S W R V R Y M V

Fig. 2. The evolutionary conservation analysis of PPP2R1A p.S256F mutation.

mutation frequency was significantly lower than that in deeply infiltrating endometriosis in the prior study (P = 0.00016) [7]. Furthermore, another prior whole-exome sequencing effort had identified ARID1A somatic mutations (p.Y454H, p.Q766X and p.P550S) in the endometriotic lesions in three out of 16 Chinese samples with ovarian endometriosis (19%, 3/16), while no mutations were identified in the PPP2R1A, KRAS and PIK3CA genes [8]. The differential mutation spectrum of the cancer driver genes between the present study and the prior report [8] indicates the complexity of ovarian endometriosis pathogenesis; alternatively, it might be caused by a relatively small sample size analyzed in the prior study. Taken together, the present study and several prior observations [7,8,19,20] strongly suggest that cancer-associated gene mutations might actively participate in the pathogenesis of ovarian endometriosis and this condition is a potential premalignant disorder.

Prior studies had revealed that ARID1A mutations frequently co-occurred with other cancer-associated gene mutations, including PIK3CA and TERT (telomerase reverse transcriptase), in diverse cancer types [21,22]. In the present study, the KRAS G12V and ARID1A Q403* mutations co-occurred in a 36-year-old sample who exhibited a high serum CA125 (308.4 U/mL) and a late age of menarche (18-year-old). To our knowledge, this is the first study to show the co-existence of KRAS and ARID1A mutations in a single individual. However, it remains unclear whether the double pathogenic mutations would be associated with the late menarche age; in addition, it is also unknown whether the double pathogenic mutations would play synergistic role in the malignant transformation of ovarian endometriosis.

In summary, we detected KRAS, PPP2R1A and ARID1A mutations in 101 Chinese samples with ovarian endometriosis; and the co-occurrence of KRAS and ARID1A mutations was identified in a single ovarian endometriosis sample for the first time. The present study, together with several prior observations, support the notion that endometriosis is a premalignant disorder.

Conflict of interest

None declared.

Acknowledgments

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